

Making an Informed Choice: Antioxidant Content Comparison Between Organic and Conventional Fruits

Determination of phenolic content in organically grown
and conventionally grown apples and blueberries using
Ultrasound assisted extraction and High-performance-
liquid-chromatography

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Organic vs. conventionally grown fruits: Which has more antioxidants?

For decades, organic foods have been a popular choice for health-conscious people. An emerging and rather popular new topic of interest today is antioxidants. Antioxidants are natural compounds found in fruits and vegetables and are said to be able to protect our cells from damage and reduce the risk of certain diseases. People concerned with health and wellness have embraced this new area and consumer products ranging from food supplements to skin care products containing antioxidants have emerged. However, whether antioxidants are as beneficial as is claimed is still debated in the scientific community.

Our research is focused on the intersection between organic food and antioxidants. The questions we want to ask are: Are organically grown fruits richer in antioxidants compared to their conventional counterparts? And if there is a difference between the two cultivation types, is it statistically significant?

To find the answers, we selected apples and blueberries to study, as these are commonly eaten fruits in Sweden. We then gathered samples of both organically and conventionally grown fruits from the market. In the lab, we freeze-dried the fruit samples to preserve the antioxidants and remove the water content. We also optimized our method for separating the antioxidants from the sample to ensure we got a high enough amount from the fruits. This was done by changing the temperature and solvent composition of the extraction process. A method called High-Performance Liquid Chromatography was used to measure the amount of antioxidants extracted. This method was also fine-tuned to successfully quantify three specific antioxidants, chlorogenic acid, naringin and quercetin-3-glucopyranoside.

We found that there was a statistically significant difference between organically and conventionally grown apples and blueberries. Organically grown apples had more antioxidants than their conventionally grown counterparts. Organically grown blueberries also had more antioxidants than the conventionally grown ones, but the difference was smaller. Our study thus suggests that if you are interested in getting more antioxidants – go organic. It however neither proves nor disproves whether antioxidants are healthy.

Abstract

Introduction: To facilitate a comparison between the polyphenolic antioxidant content of commercially grown and organically grown apples and blueberries, antioxidants were extracted using ultrasound-assisted extraction (UAE) and measured using High-performance liquid chromatography and UV-vis detection (HPLC-UV/Vis).

Background: Theories suggest that fruits and crops grown organically have more antioxidants since this is a secondary metabolite produced by plants to fight off pests. Since conventionally grown crops are given pesticides less of this secondary metabolite is needed. We wanted to see if the organically grown fruits contained more antioxidants than conventionally grown fruits. A challenge in the field is to extract antioxidants found in the fruit since these are prone to degradation. This is why our study focused on optimizing the extraction and High-performance liquid chromatography analysis.

Aim(s): The study aims to investigate and compare the difference in the antioxidant content between organic and commercial blueberries and apples.

Methods: The organic and commercial fruits were sampled and freeze-dried to remove the water. Ultrasound assisted extraction was carried out to extract the antioxidants. The extracted samples were analyzed using 2,2-diphenyl-1-picrylhydrazyl to know the total amount of antioxidants and High-performance liquid chromatography/UV-vis to quantify the present antioxidants. Ultrasound assisted extraction was optimized for solvent composition, temperature and time. As for High-performance liquid chromatography, optimization was done by changing the gradient profile and the flow rate.

Results: The optimized conditions for extracting antioxidants of interest from apples were a temperature of 55°C with a composition of 60% ethanol in water. The optimized conditions for the extraction from blueberries were at a temperature of 45°C with a composition of 50% methanol in water. For HPLC analysis, the method with the shortest analysis time of 33 minutes and a good resolution of peaks, was chosen.

Conclusion: 2,2-diphenyl-1-picrylhydrazyl proved inconclusive for our samples, while High-performance liquid chromatography/UV-Vis provided reliable in determination and quantification of the individual antioxidants. Blueberries cultivated organically exhibited higher concentrations of chlorogenic acid, quercetin-3-glucopyranoside, and naringin (37.120,

16.502, and 2.478 mg/100 g fruit respectively) compared to conventionally grown counterparts (33.120, 15.102, and 2.093 mg/100 g fruit respectively). Apples grown organically exhibited higher concentrations of chlorogenic acid (5.112 mg/100 g wet fruit) than conventionally grown ones (3.675 mg/100 g fruit respectively). Naringin concentration obtained was under detection limit. The difference was statistically significant for chlorogenic acid in apples and blueberries, and naringin in blueberries.

Keywords: antioxidants, fruits, HPLC, organic, ultrasound-assisted extraction (UAE)

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Statement of AI

AI tools were used as source of inspiration in the writing of titles and correcting grammar.

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1 List of abbreviations

α :	Separation factor	MeOH:	Methanol
ACN:	Acetonitrile	NARN:	Naringin
CA:	Caffeic Acid	N:	Plate number
CGA:	Chlorogenic Acid	P-COU:	P-coumaric acid
CV:	Correlation Coefficient	Q3G:	Quercetin-3-glucopyranoside
DAD:	Diode Array Detector	QCT:	Quercetin
DPPH:	2,2-diphenyl-1-picrylhydrazyl	ROS:	Oxygen-reactive species
		RSD:	Relative Standard Deviation
FA:	Ferulic Acid	R_s :	Resolution
GA:	Gallic Acid	t_R :	Retention Time
k:	Retention factor	SA:	Syringic acid
HPLC:	High-Performance Liquid Chromatography	SD:	Standard Deviation
LOD:	Limit of Detection	UAE:	Ultrasound-Assisted Extraction
LOQ:	Limit of Quantification	VA:	Vanillic acid

2 Introduction

By donating electrons, antioxidants neutralize free radicals. Their effectiveness in our diet has been questioned by several studies. Antioxidants protect cells and tissues to be oxidized by free radicals, which can trigger DNA mutations and carcinogenesis. This protection helps prevent certain diseases^{1,2}. However, at high concentrations, antioxidants can present a pro-oxidant activity leading to the generation of reactive oxygen species that harm cells and tissues. Polyphenol antioxidants, the most abundant type of antioxidants found in plants, have better stability and lower reactivity. This is why natural antioxidants are believed to be more beneficial than artificial antioxidant supplements³.

Research on fruits and vegetables has indicated that the antioxidant levels vary based on factors such as growing conditions. Polyphenols are produced as the plant's defence mechanisms and are stimulated under stressful conditions. Organic agriculture does not use synthetic pesticides and fertilizers, leading to the hypothesis that this agriculture may increase the polyphenol content in the fruits. This may be caused by the plant's need to protect itself against attacks from insects and pathogens, difficulty finding nitrogen sources, and low growth, hence the production of secondary metabolites, like polyphenols⁴.

Given the importance of polyphenolic compounds in protecting against various diseases, this study examines the differences in polyphenolic compound content between organically and conventionally grown fruits. Apples and blueberries are selected for this method due to their availability in stores and significant presence in our diet.

2.1 Antioxidants

Antioxidants are substances that prevent or delay the oxidation of oxidizable substrates, even when present at relatively low concentrations compared to the oxidizable substrates⁵. Natural exogenous antioxidants can be classified into 4 groups: vitamins, trace elements, carotenoids and polyphenols⁶. Polyphenols contain one or more aromatic rings and two or more hydroxyl groups. They widely exist in fruits and play an important role in reacting with free radicals. Polyphenols can be classified into five groups according to their structures: phenolic acids, flavonoids, stilbenes, lignans and tannins⁷. Phenolic acids and flavonoids in apples and blueberries were the subjects of the study. The general structures of phenolic acids and flavonoids are shown in **Figure 1**.

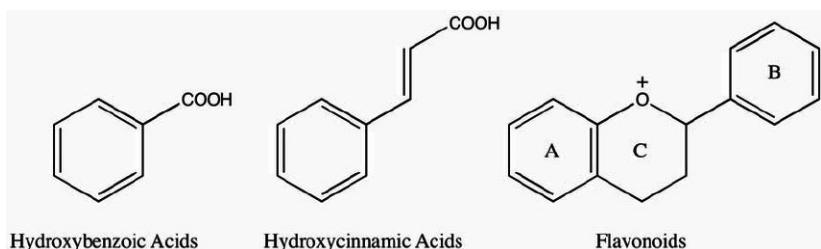


Figure 1. The basic structures of phenolic acids and Flavonoids⁸.

2.2 Summary of methodology

The general analytical strategy for antioxidant analysis involves recovery, separation by HPLC or GC, and detection using UV absorption or mass spectral methods. The antioxidant activity of extracts is widely used to determine the presence of antioxidants. However, the method is non-specific. Nevertheless, it indicates the presence of antioxidants. Due to the time constraint, samples were prepared by freeze drying followed by extraction with ultrasonication. HPLC-UV and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were used for analysis.

Representative samples of fruits were purchased from the supermarket. The sample size was reduced by following the cone and quartering method. The samples were subsequently freeze-dried with optimized timing, followed by the extraction of polyphenolic compounds using ultrasound-assisted extraction (UAE), where the temperature and solvent had been optimized. The samples were then analyzed using optimized HPLC/UV-Vis and DPPH methods. This method allowed us to compare the amount of polyphenolic compounds in the organically and commercially grown fruits.

2.3 Theory of the methods

2.3.1 Freeze-drying

Freeze-drying is a process based on the sublimation of the water present in the product used to dehydrate samples⁹. During freeze-drying, the present water in the samples is frozen, forming ice crystals by lowering the temperature. It is removed through a phase transition from ice to vapour by gradually increasing temperature under a low-pressure environment provided by vacuum¹⁰. As it operates at low temperatures, samples preserve their compounds even if they are thermolabile. Water removal aims to preserve the sample and facilitate grinding and homogenization, obtaining more representative results and helping with enhancing the extraction yield by reducing the particle size and increasing the surface area.

2.3.2 Ultrasound Extraction Technique

Ultrasound-Assisted Extraction (UAE) is an effective method using sonication to generate small vacuum bubbles, cavitation bubbles, in the solvent that collapse upon impact with the solid surface of the cell walls. The local high pressure and temperature present during the collapse facilitate the release of the cell wall content. As antioxidants are mainly present inside the plant cells and cell walls, the UAE is a suitable method to use¹¹.

It is important to use optimised extraction conditions to achieve the highest possible yields of target compounds (antioxidants). Using a higher temperature can increase the number of cavitation bubbles forming and, thus, create a larger solid-solvent contact area. This allows shorter extraction times. However, using too high temperatures can harm the stability of the extracted compounds and they rapidly degrade in the solvent. To further increase the yield, the moisture content in the sample should be minimized to enhance the solvents contact area with the solids. The solvent composition can also help in increasing yield and change the composition of the extracted compounds¹¹.

2.3.3 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method is used for measuring the total antioxidant capacity, which is the ability of the sample to catch free radicals. DPPH is a stable free radical which is purple and turns yellow when interacting with antioxidants. DPPH absorbs [photons](#) strongly at 517 nm and the absorbance decreases when the antioxidants have reacted with DPPH¹².

2.3.4 High-Performance Liquid Chromatography-UV/Visible detection

High-Performance Liquid Chromatography with UV/vis detection (HPLC/UV-Vis) is a powerful analytical tool used to separate, identify, and quantify components in a mixture. Different polyphenols exhibit peak visibility at different wavelengths. By creating a method using known analyte standards, it is possible to identify the analytes present in a sample by comparing to a standard solution at different wavelengths. Creating a calibration curve of known analytes at different concentrations enables the quantification of analytes in the sample using Beer-Lambert's law¹³.

3 Materials and Methods

3.1 Chemicals

The chemicals used are listed in Table 1.

Table 1: List of chemicals used.

CAS	Name of compound	CAS	Name of compound
149-91-7	Gallic acid	327-97-9	Chlorogenic acid
501-98-4	p-Coumaric acid	117-39-5	Quercetin
10236-47-2	Naringin	121-34-6	Vanillic acid
482-35-9	Quercetin-3-glucopyranoside	530-57-4	Syringic acid
537-98-4	Ferulic acid	331-39-5	Caffeic acid
67-56-1	Methanol (for HPLC LC-MS grade)	64-18-6	Formic acid Optima LC/MS
75-05-8	Acetonitrile (for HPLC LC-MS grade)	64-17-5	95% Ethanol (Analytical grade)

3.2 Equipment

The oven used is Termaks TS 8056. The freezer dryer used is the Labconco freezer-zone benchtop freezer 72040. The fridge (4 °C) and freezer (-18 °C) are Siemens 265299 (QC 208). The -80 °C freezer is a Thermo Scientific Forma 900. The ultrasound bath is a VWR Ultrasonic Cleaner USC – THD. The centrifuge is a Centrifuge 5804 R. The UV-Vis used for the DPPH is Shimadzu UV-1900. The analytical scale used is Sartorius Entris 224i-1S. The scale used is A&D HF-2000G. The HPLC-UV-Vis used is Agilent 1260 Infinity II. The HPLC system consisted of a diode array detector, reciprocating pump system and a reverse phase HPLC analytical column XDB-C18, 4.6 × 150 mm, 5 µm particle size. Mobile phase A was 0.1% formic acid in milli-Q water and mobile phase B was 0.1% formic acid in methanol/acetonitrile (50: 50 v/v). The Diode Array Detector (DAD) was set at 260, 280, 325 and 360 nm.

3.3 Methods

3.3.1 Determination of moisture content

Between 2 and 3 g of fruit (Ingrid Marie apples and wild blueberries from Sweden) were precisely weighed (w_1) using the analytical scale and placed into a cresol. They were covered with aluminum foil with holes and heated at 105 °C in the oven for 72 h. The samples were subsequently stored in a desiccator. Weight was taken after drying (w_2). The moisture content was calculated following **Equation 1**¹⁴.

$$MC (\%) = \frac{w_1 - w_2}{w_1} \cdot 100 \quad [\textbf{Equation 1}]$$

3.3.2 Sampling method

The cone and quartering method was used to get a representative sample while comparing different cultivation methods¹⁵. Different boxes of fruit from the same store (ICA Fäladstorget, Sweden) purchased 15th of April 2024 were mixed and quartered to reduce the sample size by 25%, quartering twice (**Figure 2**). The total blueberry sample (wild blueberries from Sweden) is 400 g and the total apple sample (Royal Gala from Italy) is 600 g.

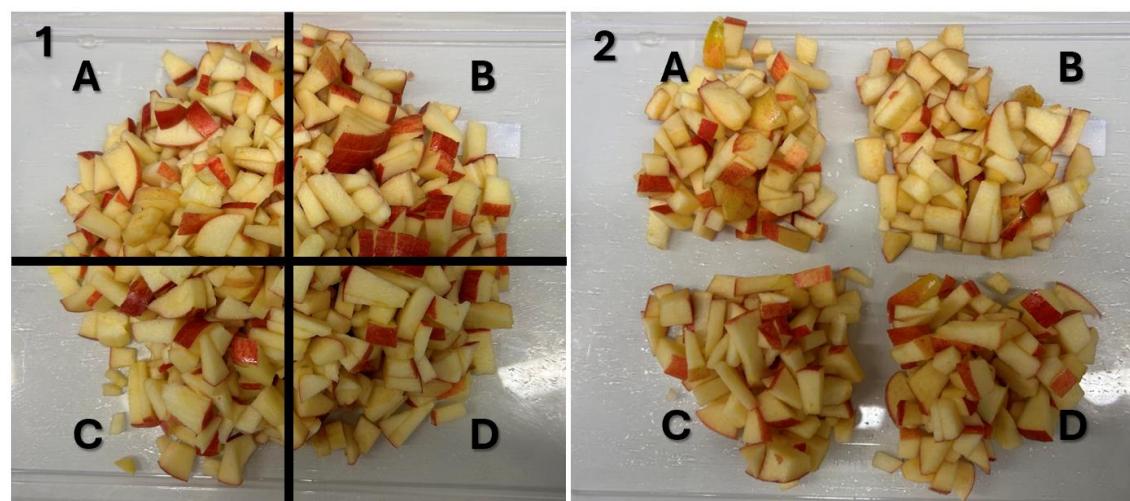


Figure 2. The quartering method used to sample fruits. Example of the apples. Step 1. Cone shaping. Step 2. Quartering. Steps 1 and 2 are repeated until the amount of sample is 100 g.

3.3.3 Freeze-drying method

The apples reduced sample (Royal Gala from Italy) and the blueberries reduced sample (wild blueberries from Sweden) were frozen for 72 h at -18 °C and then 30 min at -80 °C. They

were freeze-dried at -104 °C, 0,10 mBar for 48 hours. To optimize the method, different apple conventionally grown samples (Ingrid Marie apples from Sweden) were taken out after 5, 10, 27 and 33 hours and weighed to determine the moisture content. They were placed into the freeze-dryer in 15 mL Eppendorf tubes. Organic and conventional grown fruits (Apples Royal Gala from Italy and wild blueberries from Sweden) were compared freeze-dried directly in the flask with a hole in the middle to allow a better freeze and drying. (**Figure 3**)

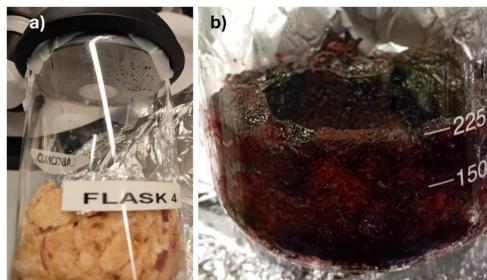


Figure 3. Fruits directly placed into the freeze-drying flask with space in the middle. **a)** apples; **b)** Blueberries.

3.3.4 Extraction method

Aliaño-González et al. 2020 utilized UAE to extract antioxidants from blueberries, while Pollini et al. 2022 also used UAE to extract antioxidants from apples^{16,17}. In this study, power, time, amount of solvent, and amount of sample were fixed parameters sourced from literature research. The optimization was based on selecting the optimal temperature and solvent composition. Two different temperatures were tested, and the best temperature was then tested with a higher percentage of organic solvent. Therefore, the temperature and solvent composition that resulted in the highest amount of antioxidants extracted could be chosen for the method by using the calibration curve gained from the HPLC-UV results to calculate the concentrations. The results obtained from the optimization step led to the following settings displayed in **Table 2**.

Table 2: List of experimental parameters for both blueberry and apple.

Sample	Solvent (V%)	Amount of solvent (mL)	Temperature (°C)	Time (min)	Power
Blueberry	45% methanol and 1% formic acid	32	45	15	7
Apple	60% ethanol and 1 % formic acid	60	55	45	7

All experiments were executed by first turning on the sonication bath and setting the right parameters: extraction time, power, and temperature. Approximately 1g of dry sample was

extracted using the appropriate solvent. The solvent and the dry sample were mixed before being placed in the sonication bath (**Figure 4**). The beakers were inspected throughout their time in the bath, and ice was added if the temperature increased by 2°C or more. The mixture was poured into a 50 ml vial and centrifuged for 40 minutes at 3800 rpm and 3°C. After the centrifugation, a pipette was used to collect the supernatant into 15 ml vials. They were transferred to 2 ml vials for HPLC. All experiments were conducted in triplicate to minimize statistical errors, with three samples being sonicated simultaneously. In literature, it is reported that antioxidants decompose upon exposure to light, the extracts were covered with aluminum foil throughout the study.



Figure 4: The picture on the right depicts weighed samples before being mixed with solvent.

The picture on the right displays the mixtures during the sonification process.

3.3.5 DPPH method

The DPPH method was used to examine the percentage of scavenging activity and measure the total antioxidant capacity of our samples of apple and blueberry antioxidant extracts. The DPPH method was based on the method described by Kubovics et. al¹⁸. 20 g of DPPH powder was measured and dissolved in 50 ml methanol. A serial dilution was carried out to obtain the concentrations of 200 ppm, 100 ppm, 50 ppm and 25 ppm. The absorbance of these concentrations was measured at wavelength 517 nm to find the concentration of DPPH to use. To measure the scavenging activity of the antioxidant sample 1 ml of DPPH solution was added to a cuvette and mixed with 1 ml of sample. The mixture was left to incubate in the dark for 30 minutes. After incubation, the absorbance of the sample was measured. The radical scavenging activity was calculated using **Equation 2**, where Abs_s is the absorbance of the sample and the value 0.709 is the absorbance of the solution of 25 ppm DPPH.

$$\% \text{ scavenging activity} = \frac{0.709 - Abs_s}{0.709} \quad [\textbf{Equation 2}]$$

3.3.6 HPLC method

The gradient program was adopted from Gondo et. al. with modification¹⁹. The aim was to ensure that compounds of interest were resolved while reducing analysis time. In this study, the standard mixture contains gallic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, quercitin-3-glucopyranoside, ferulic acid, p-coumaric acid, naringin and quercetin.

Optimization was done by changing the organic solvent percentage, in the range of 5% to 95%, and flow rate, in the range of 0.5 to 1.5 mL/min. The temperature of the column was kept as 35° C during the experiment. The optimization strategy was to reduce gaps between peaks by increasing organic solvent percentage in progressively larger increments, as well as increasing the flow rate at a specific time in the programme. In method 1 the gradient was changed and in methods 2, 3, 4 and 5 the gradient was changed further while at the same time increasing the flow rate. The working standard was a 5 ppm mixture of 10 standards dissolved in methanol. The final method used for analyzing samples is shown in **Table 3**. Identification and quantification of antioxidants in apples and blueberry extracts was done by comparison of the retention time in chromatogram with standards and calibration curves using method 4 (displayed in **Table 3**).

Table 3. Method 4 gradient profile.

Time (min)	0.1% formic acid in milli-Q water (%)	0.1% formic acid in MeOH/ACN (50: 50 v/v) (%)	Flow Rate (mL/min)
0	95	5	0.5
3	90	10	0.5
8	85	15	0.5
10.5	85	15	1
13	85	15	0.5
18	75	25	0.5
28	40	60	0.5
33	5	95	0.5

Limit of Detection (LOD) and Limit of Quantification (LOQ) were studied using linear regression for calibration curves in Excel. **Equations 3 and 4** were used, where k is the slope and SD_{intercept} is the standard error of the intercept.

$$LOD = 3.3 * (SD_{\text{intercept}} / k)$$

[Equation 3]

$$LOQ = 10 * (SD_{\text{intercept}} / k)$$

[Equation 4]

The calibration curves were obtained for ten concentrations obtained by serial dilution for the ten standards using method 4. The ten concentrations were 100 ppm, 50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm, 3.125 ppm, 1.563 ppm, 0.781 ppm, 0.391 ppm and finally 0.195 ppm. To obtain more accurate results, triplicates were made. The absorbance of the standards was measured under their optimal wavelength, which was determined by their UV spectrum (**Appendix Figure A1-10**).

3.3.7 HPLC method used for fruit samples

Identification and quantification of antioxidants in apple and blueberry extracts was done by comparison of the retention time in chromatogram with standards and calibration curves using method 4 (displayed in **Table 3**).

4 Results and discussions

4.1 Moisture content and freeze-dryer optimization

Determining the water content in the fruit before analysis is crucial for several reasons. Firstly, it allows for the accurate normalization of the antioxidant concentrations to ensure that the results are expressed consistently in dry weight. This normalization is essential because fruits can vary significantly in their water content, and comparing antioxidant levels without accounting for this variation would lead to misleading conclusions. In addition, excess water can dilute the solvent and reduce its extraction efficiency. Additionally, understanding the water content aids in the optimization of the homogenization process, as the texture and consistency of the fruit will affect how thoroughly it can be homogenized. The moisture content was calculated in triplicates for each fruit. The obtained moisture content was $87.97 \pm 0.22\%$ and $86.40 \pm 0.21\%$ for apples and blueberries, respectively.

Freeze-drying was chosen to remove water from the fruits to facilitate homogenization before extraction. The reason for the choice is that the freeze-drying process uses low temperatures, and the analytes of interest will not be degraded¹¹. The objective is to remove water to facilitate grinding the sample to result in a homogeneous powder with a high surface area after grinding the dry sample avoiding aggregation. This high surface area enhances the extraction of antioxidants prior to their separation and quantification, allowing the solvent to achieve the inside of the particles.

The freeze-dryer encountered technical problems, which resulted in some samples thawing before the set time. As a result, the results were not reproducible. In the end, the freeze-drying time was between 48 and 72 hours. The dried samples were well-ground using a pestle and mortar before extraction experiments.

4.2 Optimization of extraction method

Developing a suitable and efficient technique for extracting antioxidants is a necessary step, since extraction is the first step in evaluating the chemical composition. Numerous research works have examined various methods of extracting antioxidants from fruits^{16,17}. Based on the benefits listed in the introduction section, ultrasound-assisted extraction (UAE) was selected for this study. Optimization was limited to the extraction temperature and solvent

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composition. For apples, a mixture of ethanol and water was used while for blueberries, a mixture of methanol and water was used. The choice of solvents was based on methods from the literature. The optimized conditions for both fruits are shown in **Table 4**. Full tables showing the results for other conditions is presented in the **Appendix Table A1 and A2**.

It was concluded that the optimal conditions of the extraction of apples, in aspect to chlorogenic acid, quercetin-3-glucopyranoside and naringin acid, was a temperature of 55°C with a composition of 60% ethanol. These conditions yielded the overall highest concentration of the three selected antioxidants after extraction. Similar results were observed by Pollini et al 2022 where ethanol was mentioned as part of the solvent composition as well¹⁷. It is advantageous to use ethanol due to its polarity, even though it is less polar than methanol, making it well-suited for extracting compounds from apples. Moreover, it is preferable to use ethanol since it is non-toxic compared to other solvents. Ethanol and water work well together since ethanol enhances the rate of extraction and water-ethanol solvent allow a broad yet selective spectrum of compounds.

It was also concluded that the optimal conditions of the extraction of blueberries, with respect to chlorogenic acid, quercetin-3-glucopyranoside and naringin acid, was a temperature of 45°C with a composition of 50% methanol. The results are not far off from those observed by Alliano-Gonzalez et al 2020 in their experiments on blueberries, which we used similar conditions¹⁶. Methanol is employed in these experiments since studies has proven it to have high effectivity in total antioxidants-and total phenolic content extractions. Although, methanol has been classified as class 2 according to FDA (Food and Drug Administration) due to the toxicity and the health consequences when it is consumed. Nevertheless, it is extensively recommended for analytical purposes due to its polarity, viscosity and small size, which makes it very easy to penetrate cell membranes. These conditions gave the overall highest concentration of the three selected antioxidants after extraction.

As our method used fixed extraction times taken from literature it is not fully known if the extraction was fully completed in our case. If the extraction is not completed, then the concentration profile is steeper, and the results will have a more significant margin of error compared to a completed extraction where the concentration profile has stagnated. This could be the reason for the high standard deviations presented in **Appendix Table A1 and A2**.

Table 4. Optimized conditions for extraction of antioxidants from apples and blueberries. Extraction time was constant at 45 minutes for apples and 15 minutes for blueberries. The extraction temperature chosen for apples was 55°C and for blueberries 45°C. Solvent composition determined for apples was 60% ethanol while 45% methanol for blueberries. The experiments were done in triplicates and the measured average concentration for CGA, Q3G and NARN are displayed.

Sample	Extraction temperature (°C)	Solvent composition (v/v)	Average Concentration (µg/ml) ± SD (RSD)	
Apples	55	60 % ethanol in water	CGA	1.75 ± 0.03 (1.52)
			Q3G	0.38 ± 0.00 (1.17)
			NARN	1.01 ± 0.04 (3.47)
Blueberries	45	45 % methanol in water	CGA	66.97 ± 0.87 (1.30)
			Q3G	43.71 ± 0.40 (0.92)
			NARN	5.30 ± 0.08 (1.47)

4.3 Optimization of DPPH

DPPH is a well-established method to measure the antioxidant activity in fruits¹⁸. Therefore, our first approach was to determine the DPPH concentration for measuring antioxidant activity during extraction method optimization. We used different concentrations of the standard mix to determine which would be the most effective. Modification of the method described by Kubovics et al 2018¹⁸ was used and a concentration of 25 ppm was found to be suitable as it had an absorbance of 0.709. The results of other concentrations tested are listed in **Appendix Table A3**.

Preliminary results from the first set of extraction experiments are shown in **Table 5**. Blueberries showed a higher absorbance than apples. As blueberries have such a high average absorbance the activity obtained resulted in a negative value. We believe the dark colour of the blueberry extract interferes with the absorbance, and the method might work by using a more diluted sample. Nevertheless, the results are not surprising as it has already been reported in the literature that blueberries have more antioxidants than apples. The disadvantage of the DPPH assay is that it is not specific. Therefore, it is not possible to report which antioxidants are present. Thus, due to the limitation of time allocated to perform experiments, HPLC-UV was used for further extraction method development.

Table 5: The average activity obtained using a reference absorbance of 0.709. Extraction time was constant at 45 minutes for apples and 15 minutes for blueberries. The extraction temperature chosen for apples is 55°C and for blueberries 45°C. Solvent composition determined for apples was 60% ethanol while 45% methanol for blueberries. The experiments were done in triplicates and the average absorbance, dilution factor and average activity were measured and are displayed.

Extraction sample	Solvent composition (v/v)	Extraction temperature (°C)	Average absorbance ± SD	Dilution factor	Average activity (%)
Apples	45% ethanol in water	45	0.07 ± 0.01	1	0.90
Apples	45% ethanol in water	55	0.09 ± 0.04	1	0.88
Blueberries	45% methanol in water	35	2.32 ± 0.04	2	-4.54
Blueberries	45% methanol in water	45	2.29 ± 0.04	2	-4.45

4.4 Optimization of HPLC-UV/Vis

4.4.1 Optimization of the method

Chromatograms for five methods across four wavelengths are shown in **Figure 5**. The aim of the optimization was to shorten the analysis time while maintaining a good peak separation. In **Appendix Tables 4, 5, 6 and 7** we display the five methods we tried when optimizing the HPLC method. To accelerate the elution of all peaks, the first method involves increasing the gradient slope within the first 0–10-minute span. This resulted in an analysis time of 46 minutes. In the second method, the flow rate was increased during the 15–20 min span to shorten the gap between chlorogenic acid and vanillic acid. This led to an analysis time of 40 minutes. As for the third method, it combines the alterations from the first two methods, giving an analysis time of 35 minutes. The fourth method further reduced the gap between gallic acid and chlorogenic acid and had an analysis time of 33 minutes. Method five aimed to reduce the gap between p-coumaric and ferulic acid and had an even shorter analysis time of 31 minutes. Still, it was discarded due to the overlapping of the mentioned peaks. In the end, a compromise was made to use a longer method with good resolution, using method 4 in the following experiments.

The elution order of the ten standards is determined by their size, polarity, functional groups, log P values and pKa values. We see that the more polar compounds elute first when we have a higher water concentration, and the less polar compounds elute last when the organic

solvent concentration is increased according to the elution gradient. The structures of the ten analytical standards used can be seen in **Appendix Figure A11**.

Figure 5 shows that the peaks have different sizes even though they all have the same concentration. This is caused by the different sensitivities of the compounds to the UV due to the molar extinction coefficient in Beer-Lambert's law. We also see some peaks fronting, namely peaks 3, 4 and 5 and to a lesser extent also peaks 6, 7, 8 and 9. The fronting could be caused by the flow rate which is increased at 17.5 in method 2, 12.5 in method 3 and 10.5 minutes in methods 4 and 5. However, the flow rate is not increased in method 1 where we also see peak 6 fronting. This may be explained by the impurities present. Impurities could be checked by performing HPLC-MS, but this was not done because of time constraints.

In **Table 7** the retention time, retention factor, separation factor, plate number and resolution for each compound are displayed for method 4. As seen from the table, all resolution values are above 2, indicating a good peak separation and thus allowing for the identification of antioxidants.

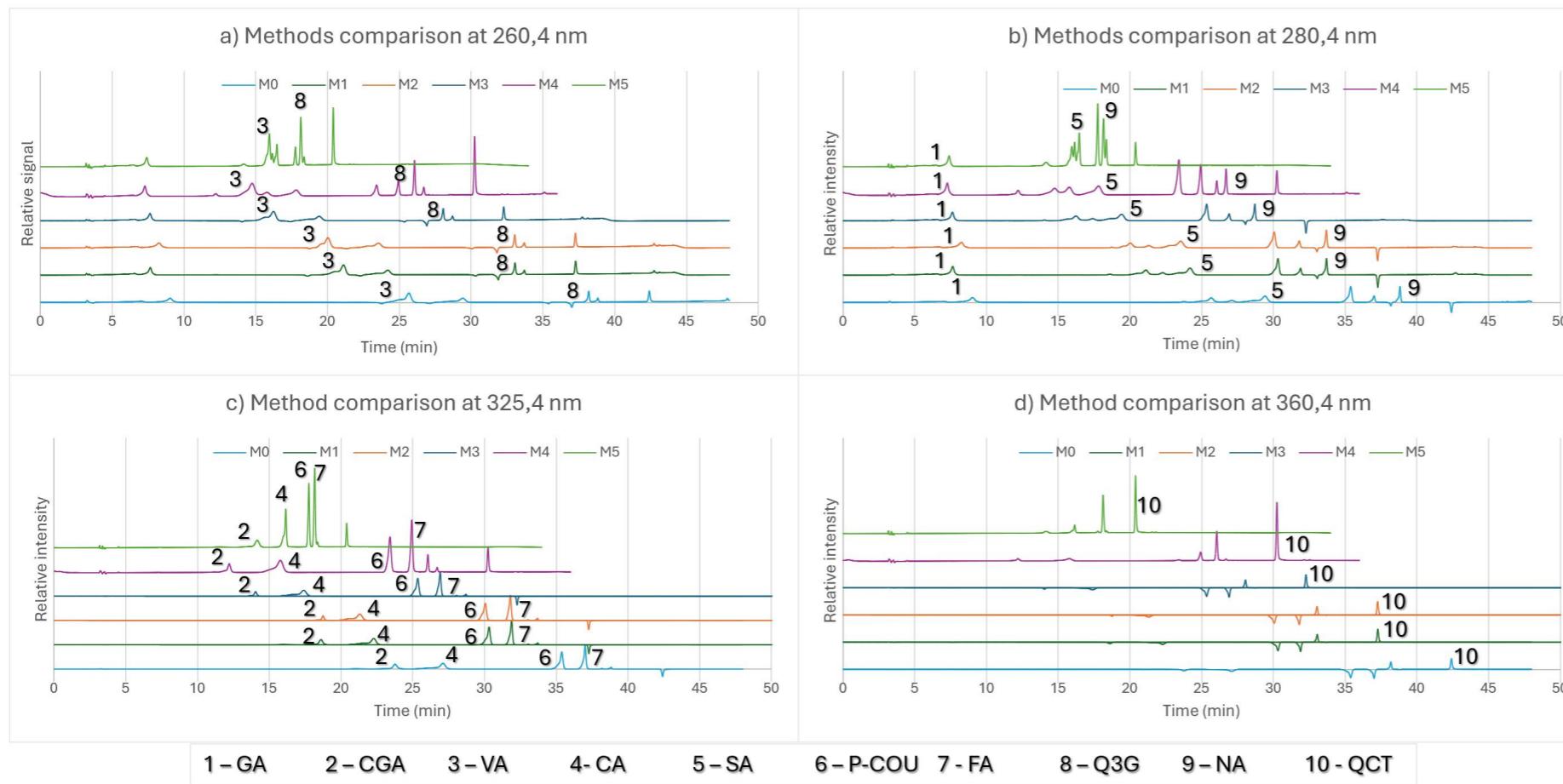


Figure 5. Chromatograms of a mixture with a concentration of 5 ppm for each standard with different methods at different detection wavelengths.

a) 260.4 nm; b) 280.4 nm; c) 325.4 nm and c) 360 nm.

Table 6: Peaks obtained using the 5ppm standard mix of the ten antioxidant standards for identification of compounds in apples and blueberries, where RT is retention time, k is the retention factor, α is the separation factor, N is the plate number and Rs is the resolution.

Peak no.	t _R (min)	Compound	Wavelength (nm)	k	α	N	Rs
1	7.29	GA	280	1.268	---	854.214	3.107
2	12.22	CGA	325	2.802	2.209	564.539	2.098
3	14.79	VA	260	3.603	1.286	1693.291	4.375
4	15.84	CA	325	3.929	1.091	775.476	2.957
5	17.86	SA	280	4.558	1.160	1975.847	4.726
6	15.62	P-COU	325	6.294	1.381	10103.820	10.684
7	24.95	FA	325	6.762	1.075	8447.110	7.982
8	26.06	Q3G	260	7.109	1.051	47006.668	23.050
9	26.71	NARN	280	7.309	1.028	66014.154	27.314
10	30.25	QCT	360	8.412	1.151	14982.164	13.009

4.4.2 Calibration of antioxidants standards

Figure 6 and **Table 7** show the calibration curve results for the 10 standards and their LOQ and LOD. The determined coefficient R^2 is 0.999 for all the fitting equations, indicating a good linear relationship. However, the residual plot in **Figure 7** shows variance among data points, and the error variance increases with concentration, so it can be estimated that only medium and low concentration fit in the linear range. We can see that gallic acid, ferulic acid, P-coumaric and caffeic acid have a curved residue plot which indicates that the linear fit is not good, while the rest of the standards have a random plot which indicates a good fit. It can also be noted that standard deviations among triplicates are relatively high, which can be related to the reproducibility of the HPLC system and random error during pipetting of solutions during dilutions. These factors can influence the detected LOD and LOQ. The calculated detection limit for quercetin-3-glucopyranoside is the lowest among the tested standards, indicating its capability for detection at low concentrations. Conversely, chlorogenic acid exhibits the highest LOD. Regarding the calibration curve, the slope implies different sensitivities among the compounds, due to the different UV sensitivity which is caused by the molar extinction coefficient in Beer-Lambert's law. The sensitivity of used compounds is ranking as follows: ferulic acid > p-coumaric acid > caffeic acid > vanillic acid > quercetin > syringic acid > quercetin-3-glucopyranoside > gallic acid > naringin > chlorogenic acid.

The calculated LOD and LOQ are higher than the typical range of 0.1 – 3 ppm reported in other studies, but generally in an acceptable range²⁰.

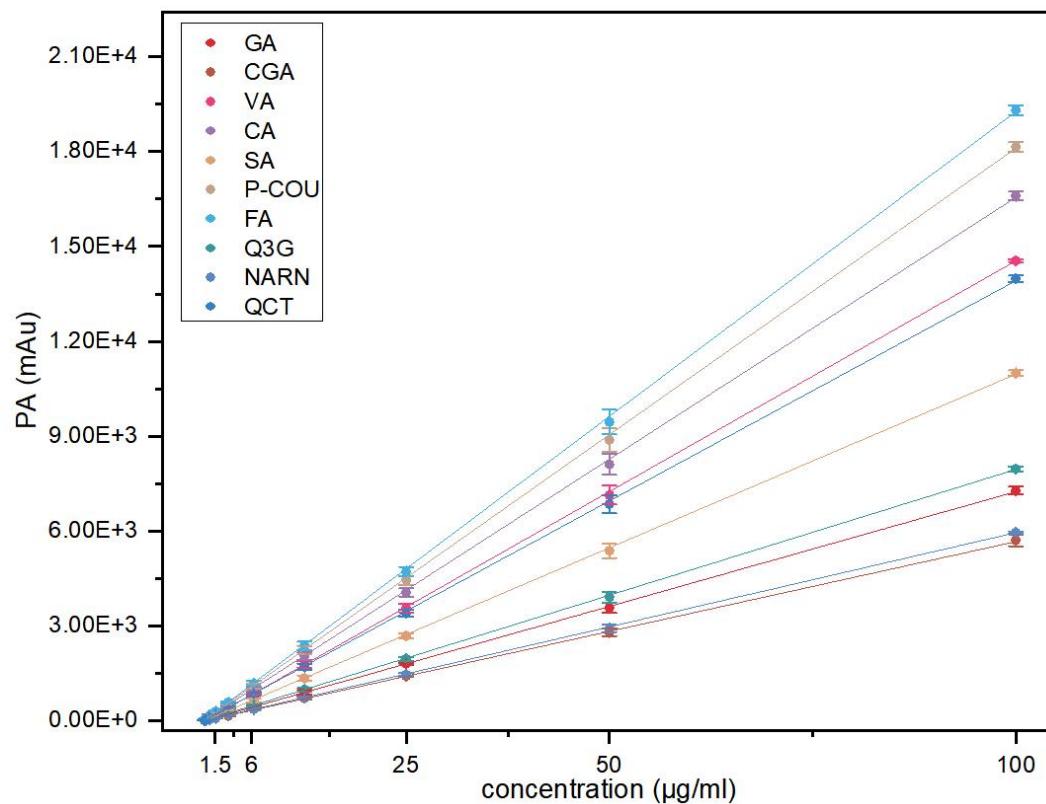


Figure 6. Calibration curves for the standards.

Table 7. Regression equation parameters for 10 standards, with LOD and LOQ calculated from standard error of equation slope.

Antioxidant	Slope	Intercept	R ²	LOD (µg/ml)	LOQ (µg/ml)
GA	72.527 ± 0.191	-3.270 ± 1.310	0.999	1.702	5.159
CGA	56.748 ± 0.627	-3.316 ± 2.912	0.999	1.975	5.984
VA	145.823 ± 0.401	-38.605 ± 7.800	0.999	1.751	5.305
CA	165.432 ± 0.549	3.901 ± 4.314	0.999	1.971	5.972
SA	109.663 ± 0.560	-7.139 ± 4.217	0.999	1.907	5.778
P-COU	181.029 ± 0.528	1.293 ± 0.783	0.999	1.557	4.719
FA	192.603 ± 0.551	0.402 ± 0.776	0.999	1.551	4.700
Q3G	79.506 ± 0.205	1.177 ± 0.366	0.999	1.447	4.383
NARN	59.522 ± 0.099	0.731 ± 0.389	0.999	1.537	4.656
QCT	139.193 ± 0.528	-3.347 ± 0.861	0.999	1.473	4.463

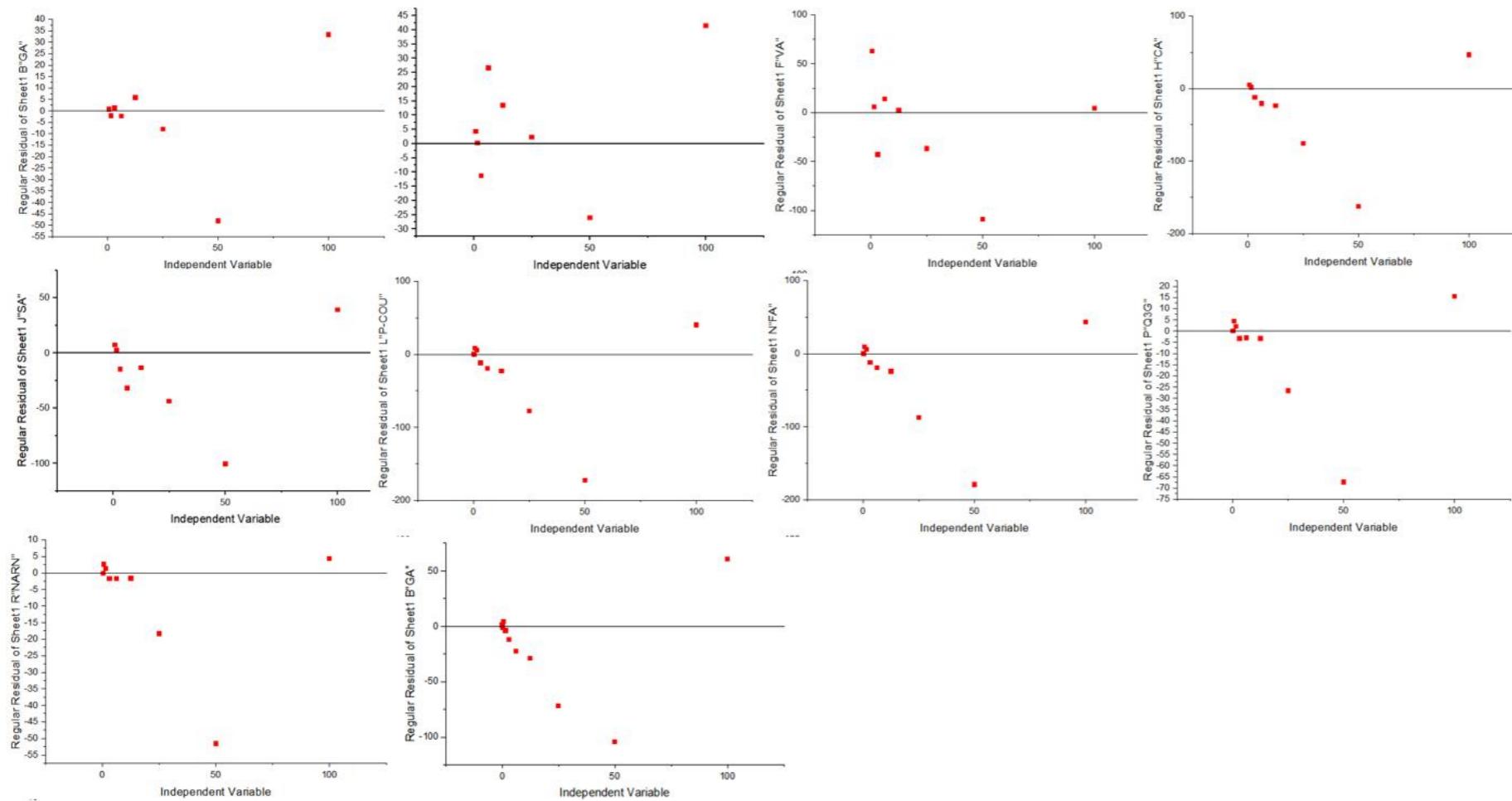


Figure 7. Residual plot for standards.

4.5 Comparison of commercial and organic apples and blueberries

The chromatograms obtained for the samples are presented in **Appendix Figure A12, A13, A14 and A15**. We can see that the same chromatogram is obtained for the triplicates done, showing good reproducibility of the method. Comparisons of the obtained chromatograms and the standard mixture are presented in **Figures 8 and 9**, where we can see that only chlorogenic acid, quercentin-3 glucopyranoside and naringin were found on organically and commercially grown apples and blueberries. We see from the figures that the baseline of the blueberry sample is not good. There seems to be a lot of matrix interference with the peaks. This leads to the quantification of antioxidants being less precise. The baseline is better for the apple extracts, and the peaks can be clearly distinguished from the matrix. With the apple samples we, however, have the limitation of some analytes not being above the LOQ which compromises our conclusions.

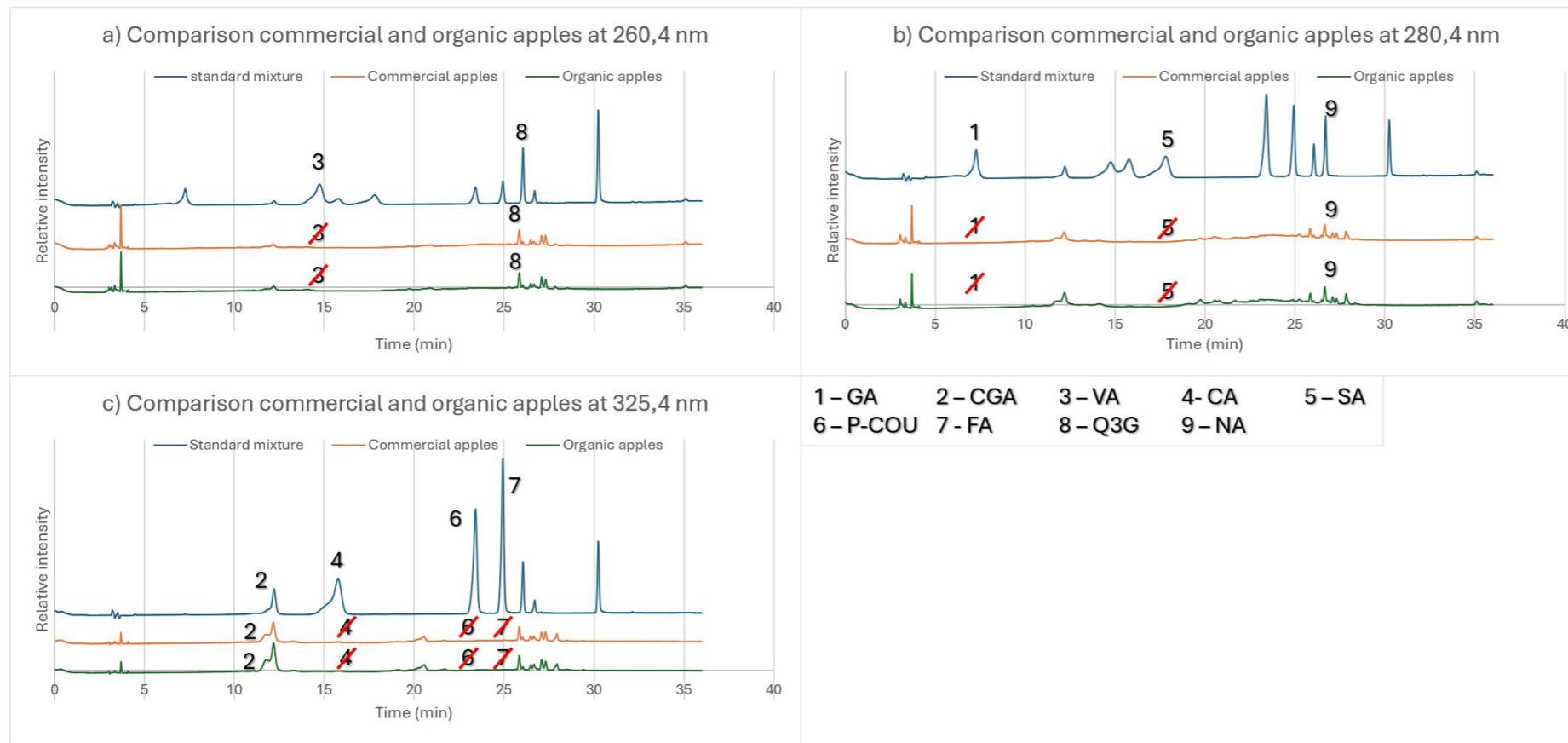


Figure 8. Chromatograms obtained by commercially and organically grown apples compared to the standard mixture. Numbers show the different analytes.

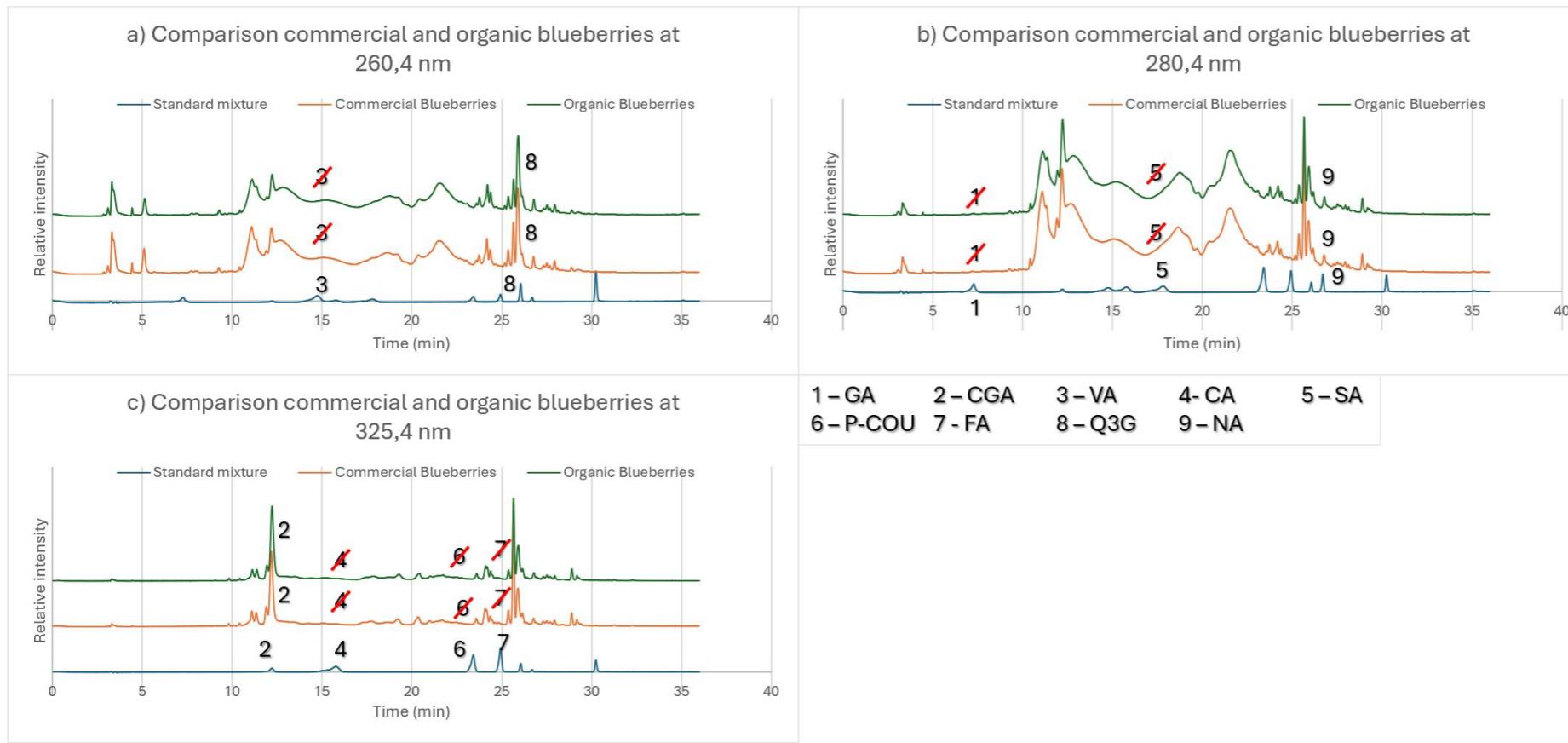


Figure 9. Chromatograms obtained by commercially and organically grown blueberries compared to the standard mixture. Numbers show the different analytes.

Table 8: Concentrations obtained when analyzing commercial and organic **apple** samples.

$p > 0.05$ for Q3G and NARG. $p < 0.05$ for CGA. FW stands for wet fruit, SD for standard deviation and RSD for relative standard deviation, * indicates concentration lower than LOQ.

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Compound	Fruit type	Average Concentration in extracted solution ($\mu\text{g}/\text{ml}$)	Average Concentration in fruit (mg/100 g FW)	SD	RSD (%)	p-value
CGA	Commercial	4.009	3.675	0.011	0.306	1.082 E-06
	Organic	5.777	5.112	0.040	0.789	
Q3G	Commercial	0.302*	0.277*	0.024	8.895	0.371
	Organic	0.283*	0.250*	0.027	10.771	
NARN	Commercial	1.418*	1.210*	0.045	3.485	0.460
	Organic	1.512*	1.342*	0.057	4.238	

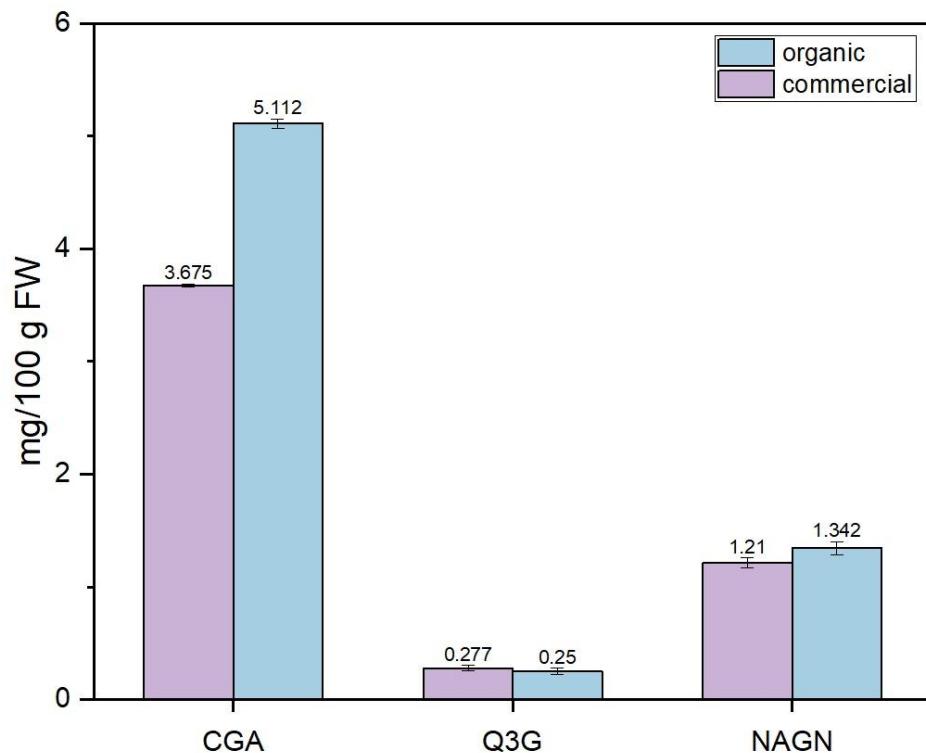


Figure 10: Bar chart showing the quantification of antioxidants in apples.

Table 9: Concentrations obtained when analyzing commercial and organic blueberry samples. $p < 0.05$ for CGA and NARG. $p > 0.05$ for Q3G. FW stands for wet fruit, SD for standard deviation and RSD for relative standard deviation.

Compound	Fruit type	Average Concentration in extracted solution ($\mu\text{g}/\text{ml}$)	Average Concentration in fruit ($\text{mg}/100 \text{ g FW}$)	SD	RSD (%)	p-value
CGA	Commercial	79.353	33.120	0.360	1.334	0.0201
	Organic	84.021	37.377	1.568	4.196	
Q3G	Commercial	36.173	15.102	0.173	1.406	0.136
	Organic	37.089	16.502	0.797	4.829	
NARN	Commercial	5.014	2.093	0.025	1.174	0.007
	Organic	5.570	2.478	0.105	4.240	

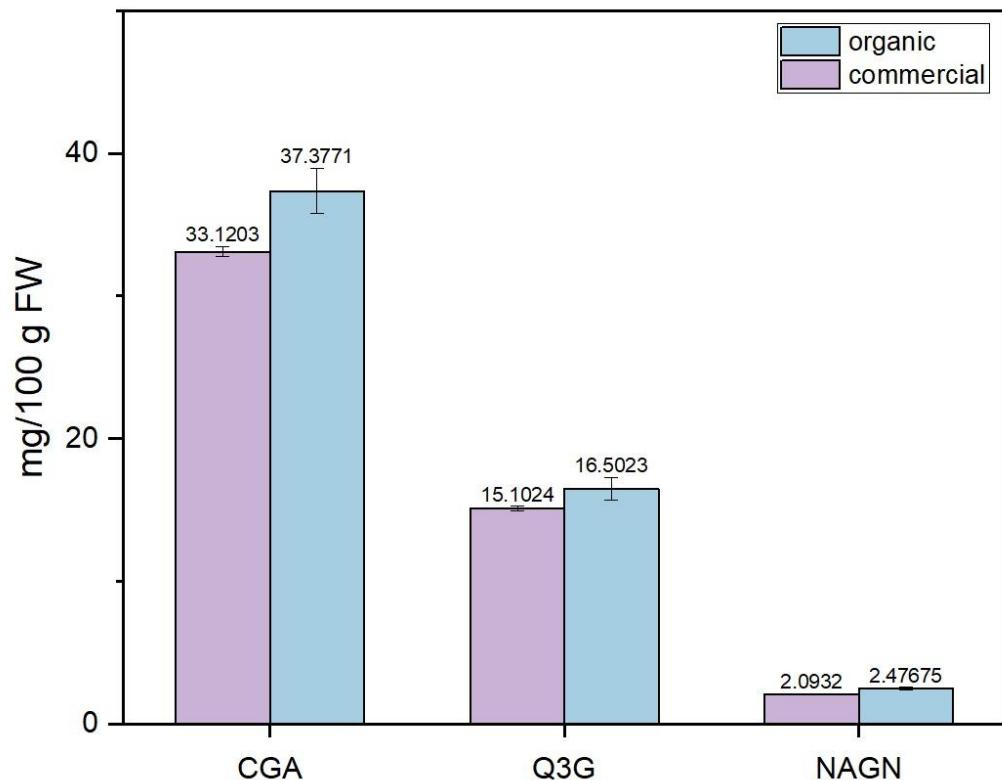


Figure 11: Bar chart showing the quantification of antioxidants in blueberries.

The calculated concentrations of analytes in the fruit samples from the obtained chromatograms are shown in **Tables 8 and 9** and have been visualised in bar charts in **Figure 10 and Figure 11**. Those values give similar results to the values found by A. Loncaric et. al. for chlorogenic acid and quercetin-3-glycopyranoside in apples and by N. Liovic et.al. for chlorogenic acid in blueberries^{21,22}. A higher antioxidant concentration was found in

organically grown fruits, confirming that those growing conditions enhance the plant to produce more secondary metabolites, e.g. antioxidants. However, it is noted that the concentration of Q3G and NARN in apples is lower than the calculated LOD and LOQ, which means the result might not be completely reliable.

Student t-test was done to determine the statistical significance of the difference between organic and commercial fruits. The p-values are displayed in **Tables 8 and 9**. A p-value larger than 0.05 was obtained for apples for quercetin-3-glucopyranoside and naringin, indicating no significant difference in the analyte content between both cultivation methods. On the contrary, a significant difference can be observed for chlorogenic acid. Regarding blueberries, chlorogenic acid and naringin had a p-value lower than 0.05, indicating a significant difference between the samples. Quercetin-3-glucopyranoside had a p-value larger than 0.05, indicating no differences between both samples. **Table 10** shows a summary of the differences found²³.

It is important to note that the differences we see could have been caused by other factors than purely the cultivation methods of the apples and blueberries. There could be variations in soil quality and temperature where the apples and blueberries were grown. Regarding the sampling method, we used the cone and quartering method to obtain a representative sample chosen randomly and minimize the sampling error. More batches could be analysed to ensure that the significant differences depend on the growing method of the fruit, but this was not done due to time constraints. Treating organic and commercial fruit with the same procedures made the systematic errors the same in both types of fruit, mainly depending on random errors while comparing the organically and commercially grown fruits.

Table 10. Summary of differences found in organically and commercially grown fruits.

	Apples	Blueberries
CGA	Significant differences	Significant differences
Q3O	No significant differences	No significant differences
NARG	No significant differences	Significant differences

5 Conclusions

We have found that DPPH is not a reproducible method for calculating antioxidant content with our extracted samples. On the other hand, UAE followed by HPLC-UV/Vis has been a reproducible method to determine the phenolic content of specifically chosen antioxidants in freeze-dried apples and blueberries. Organically grown apples and blueberries were found to contain more chlorogenic acid, quercetin-3-glucopyrosid and naringin than conventionally grown.

6 Future aspects

Since our study was limited to comparing conventionally grown apples and blueberries, it could be interesting to broaden the search and see if differences also exist between antioxidant levels in other conventionally and organically grown fruits and vegetables.

Moreover, it could be interesting to identify the optimal farming practices for different crops to maximize the antioxidant content and minimize the environmental impact. Our study shows that organically grown apples and blueberries have a statistically significant higher antioxidant content than conventionally grown blueberries and apples, so this could be a reason for focusing more on organic cultivation methods.

Taking a more interdisciplinary approach, it may also be interesting to assess the long-term health effects of consuming these differently grown fruits. Tracking health outcomes based on dietary habits over time could give a better understanding of the effects of growing crops conventionally or organically. Furthermore, research on the bioavailability of antioxidants in organically grown and conventionally grown fruits could be of scientific use to improve diet recommendations.

Lastly it can be of interest to consider the geographical and climate factors that influence the antioxidant content in fruits. This could be done by doing comparative studies across regions.

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8 Appendix

8.1 Standard antioxidants UV spectra

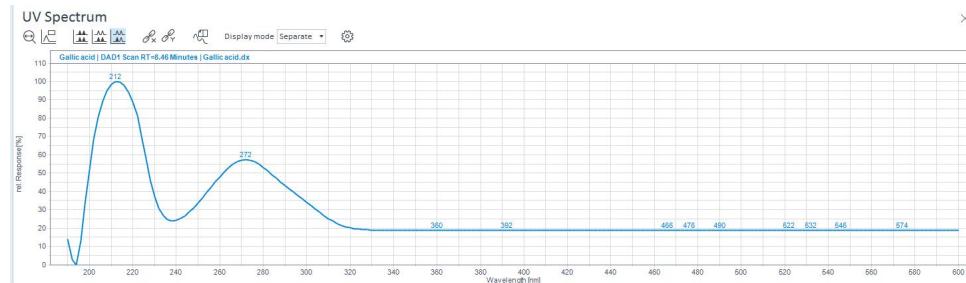


Figure A1. Gallic acid

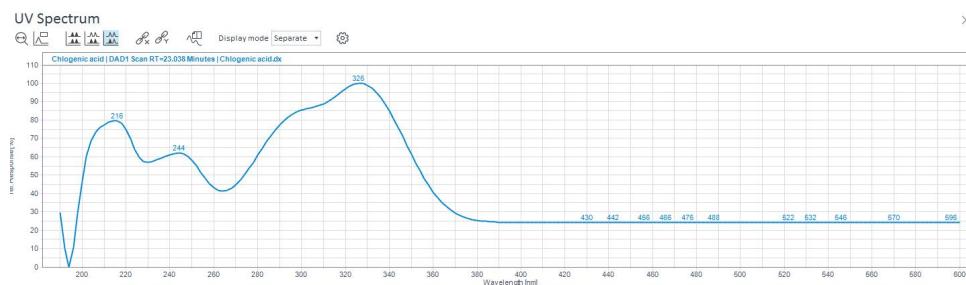


Figure A2. Chlorogenic acid

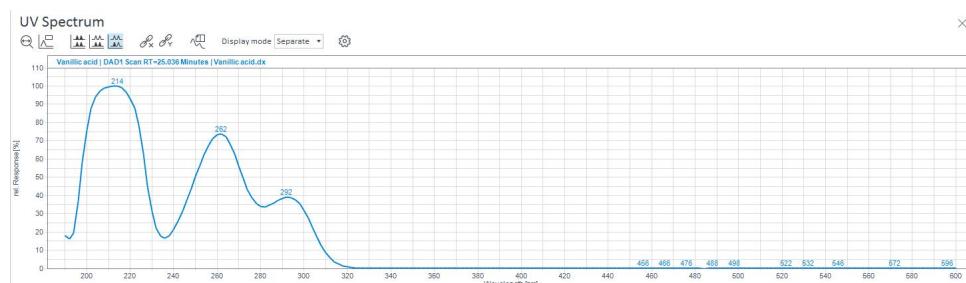


Figure A3. Vanillic acid

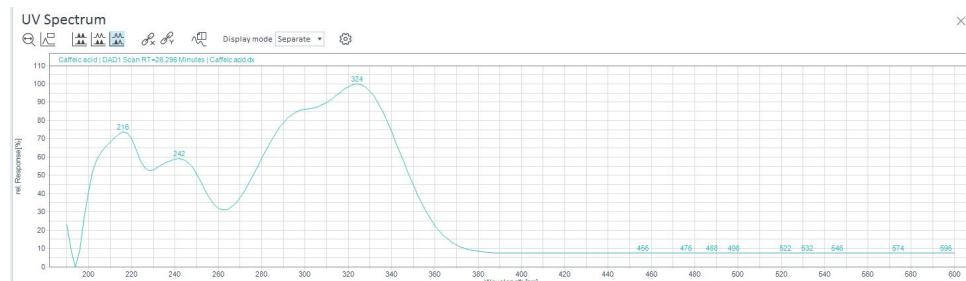


Figure A4. Caffeic acid

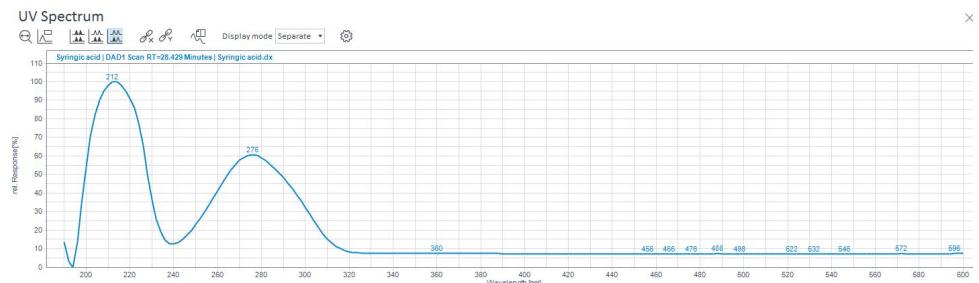


Figure A5. Syringic acid

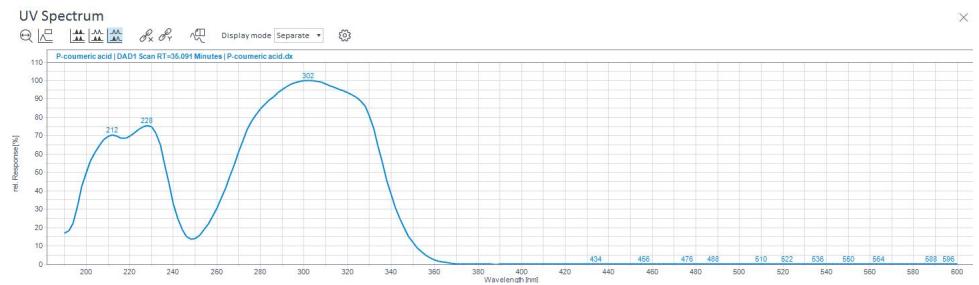


Figure A6. P-coumaric acid

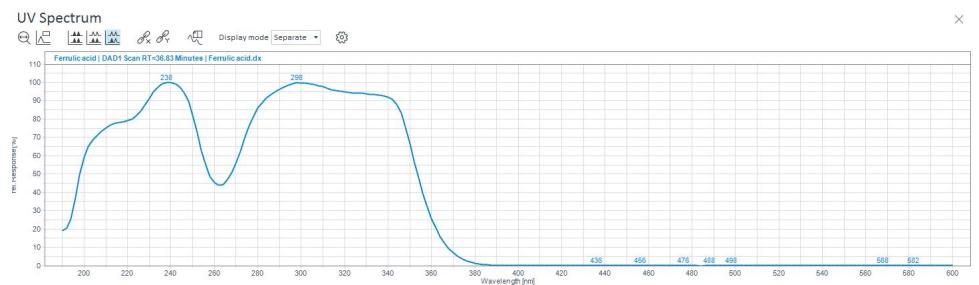


Figure A7. Ferulic acid

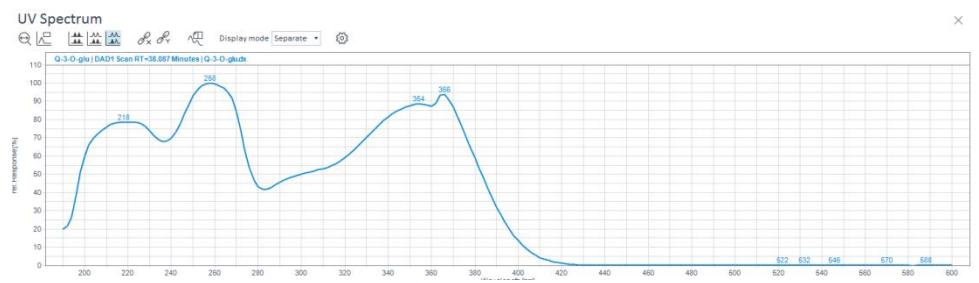


Figure A8. Quercetin-3-glucopyranoside

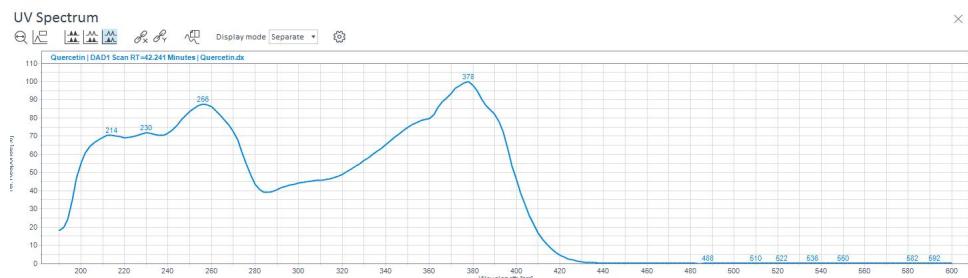


Figure A9. Quercetin

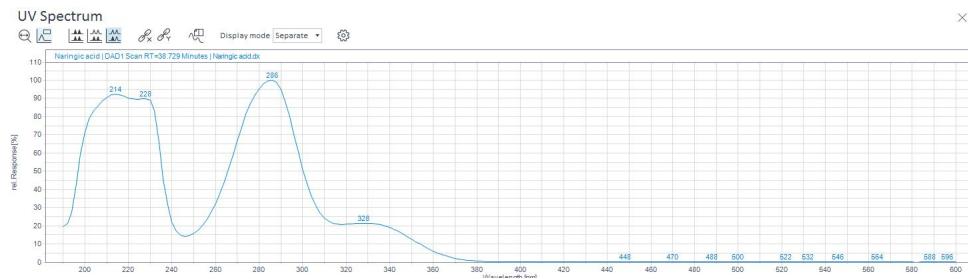


Figure A10. Naringin

8.2 Extraction optimization method

Table A1: Concentrations obtained when optimizing extraction for *apple* samples.

Extraction temperature (°C)	Solvent composition	Compound	Average Concentration (µg/ml)	SD	RSD (%)
45	50% EtOH	CGA	1.486	0.119	7.990
55	50% EtOH		1.541	0.054	3.490
55	60% EtOH		1.746	0.026	1.516
45	50% EtOH	Q3G	0.299	0.026	0.774
55	50% EtOH		0.369	0.030	8.146
55	60% EtOH		0.381	0.004	1.171
45	50% EtOH	NARN	0.852	0.052	6.074
55	50% EtOH		0.892	0.034	3.763
55	60% EtOH		1.012	0.035	3.474

Table A2: Concentrations obtained when optimizing extraction for blueberry samples.

Extraction temperature (°C)	Solvent composition	Compound	Average Concentration (µg/ml)	SD	RSD (%)
35	45% MeOH	CGA	59.683	4.869	8.159
45	45% MeOH		66.972	0.867	1.295
45	60% MeOH		58.901	0.211	0.359
35	45% MeOH	Q3G	39.383	1.641	4.168
45	45% MeOH		43.710	0.402	0.920
45	60% MeOH		43.683	0.881	2.017
35	45% MeOH	NARN	4.824	0.235	4.864
45	45% MeOH		5.303	0.078	1.474
45	60% MeOH		5.010	0.071	1.411

8.3 DPPH optimization results

Table A3: Showing the absorbance values for three different standard mixture concentrations for the different DPPH concentrations.

DPPH concentration (uM)	ABS (10 ppm standard)	ABS (5 ppm standard)	ABS (1 ppm standard)
50	0.024	0.002	0.036
100	-0.021	0.067	0.177
150	-0.04	0.072	0.416
200	0.055	0.098	0.528
250	-0.021	0.256	0.724
300	0.03	0.402	1.041

8.4 HPLC optimization method

Table A4. Description of conditions used in method 1 (M1).

Time (min)	0.1% formic acid in milli-Q water (%)	0.1% formic acid in MeOH/ACN (50: 50 v/v) (%)	Flow Rate (mL/min)
0	95	5	0.5
5	95	5	0.5
10	90	10	0.5
15	85	15	0.5
25	85	15	0.5
30	75	25	0.5
40	40	60	0.5
45	5	95	0.5
46	95	5	0.5

Table A5. Description of conditions used in method 2 (M2).

Time (min)	0.1% formic acid in milli-Q water (%)	0.1% formic acid in MeOH/ACN (50: 50 v/v) (%)	Flow Rate (mL/min)
0	95	5	0.5
5	95	5	0.5
10	90	10	0.5
15	85	15	0.5
17.5	85	15	1.5
20	75	25	0.5
25	40	60	0.5
35	5	95	0.5
40	90	5	0.5

Table A6. Description of conditions used in method 3 (M3).

Time (min)	0.1% formic acid in milli-Q water (%)	0.1% formic acid in MeOH/ACN (50: 50 v/v) (%)	Flow Rate (mL/min)
0	95	5	0.5
5	90	10	0.5
10	85	15	0.5
12.5	85	15	1.5
15	75	25	0.5
20	40	60	0.5
30	5	95	0.5
35	90	5	0.5

Table A7 Description of conditions used in method 5 (M5).

Time (min)	0.1% formic acid in milli-Q water (%)	0.1% formic acid in MeOH/ACN (50: 50 v/v) (%)	Flow Rate (mL/min)
0	95	5	0.5
3	90	10	0.5
8	85	15	0.5
10.5	85	15	1.5
11	75	25	0.5
16	40	60	0.5
26	5	95	0.5
31	95	5	0.5

8.5 Antioxidant structures

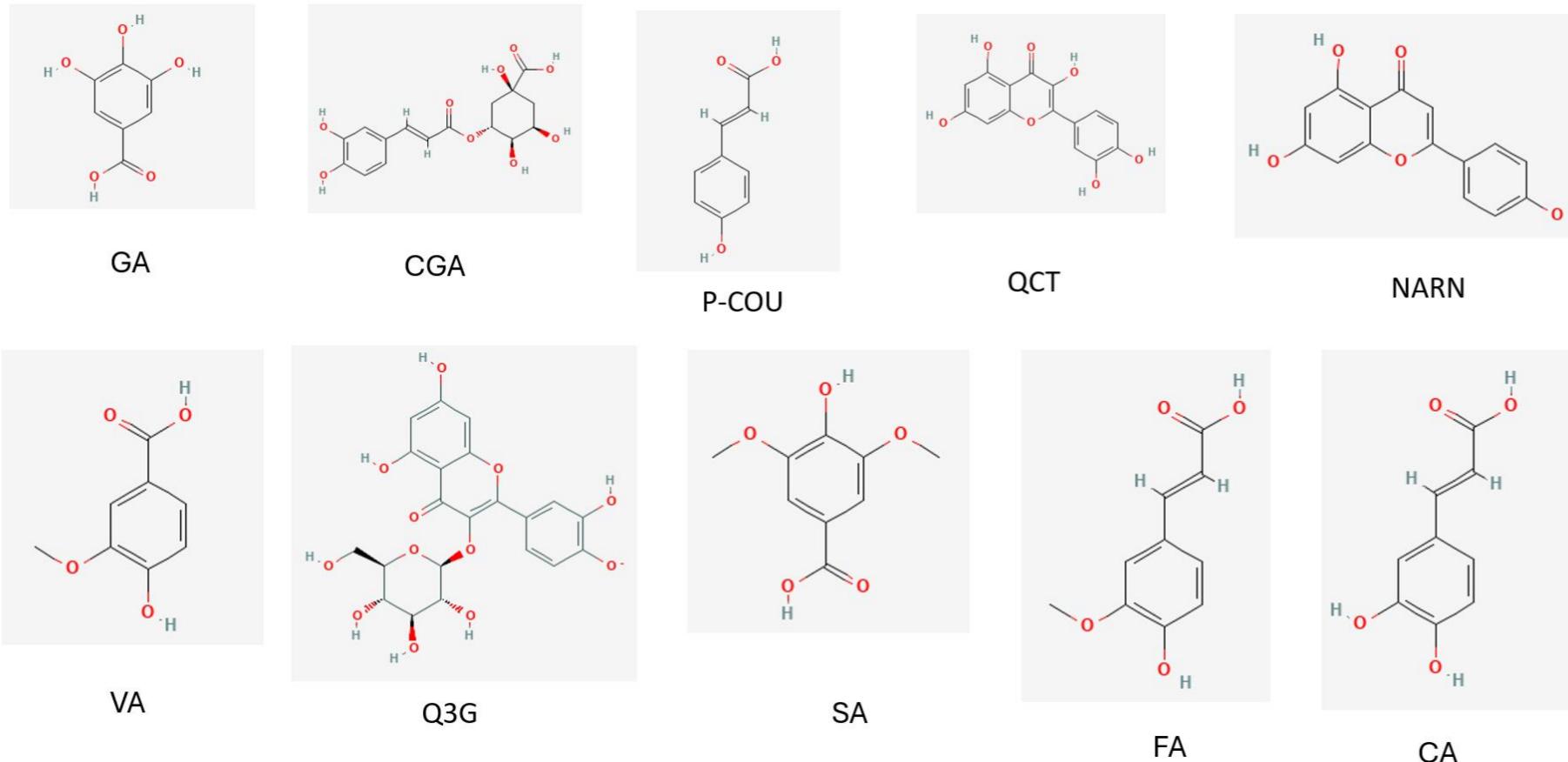


Figure A11. Structures of the ten antioxidant standards used to develop the method.

8.6 Samples chromatograms

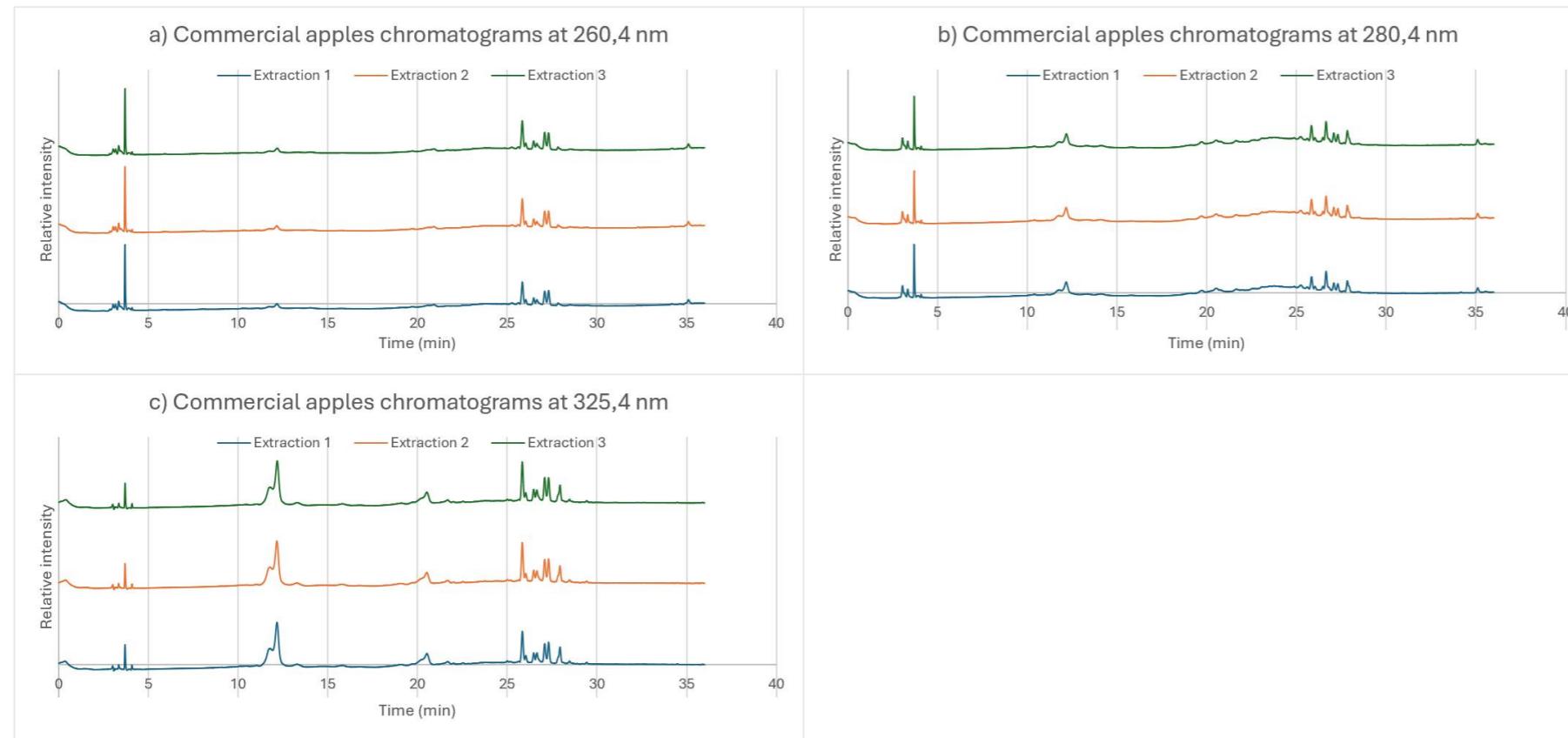


Figure A12. Obtained chromatograms for the 3 extractions done in commercial apple sample.

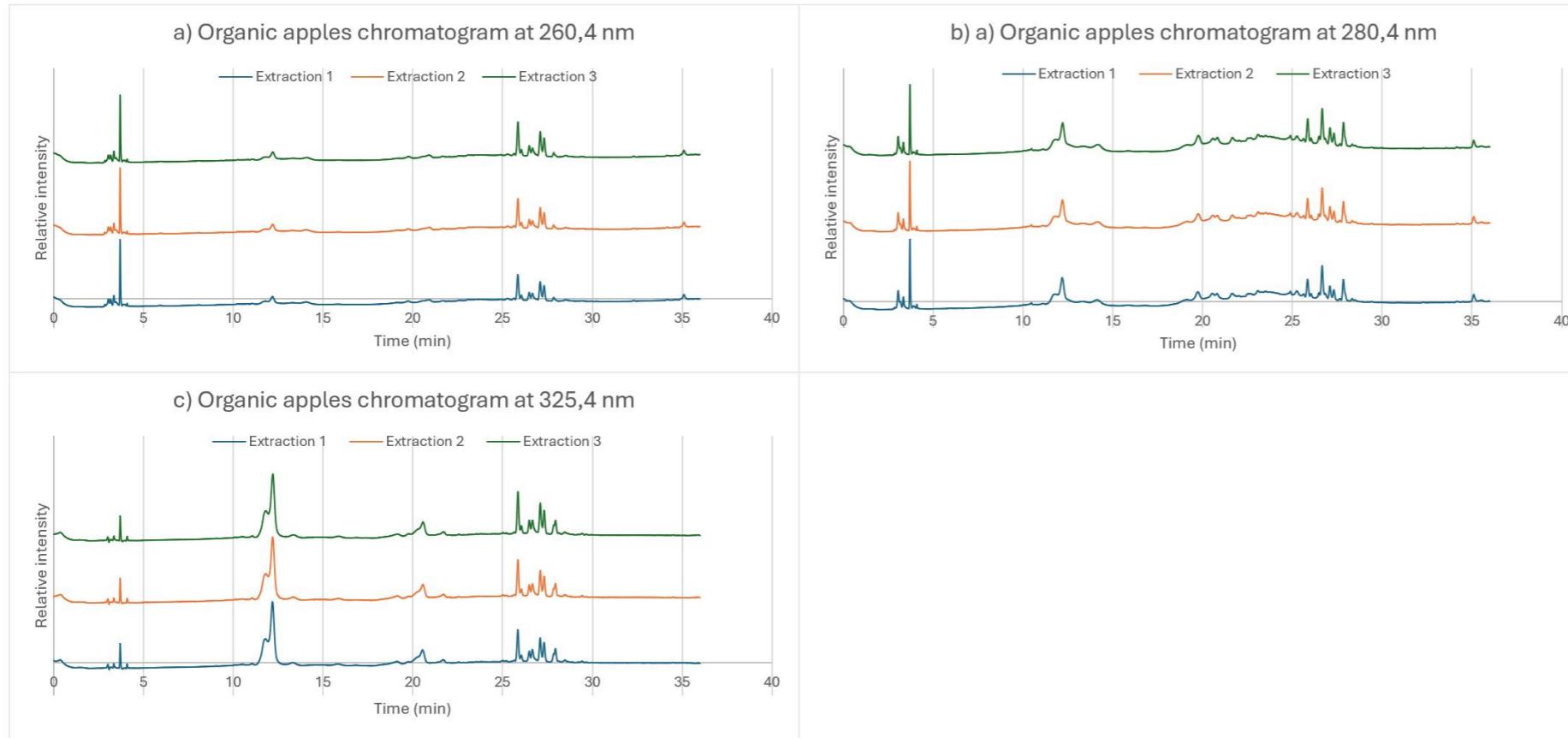


Figure A13. Obtained chromatograms for the 3 extractions done in organic apple sample.

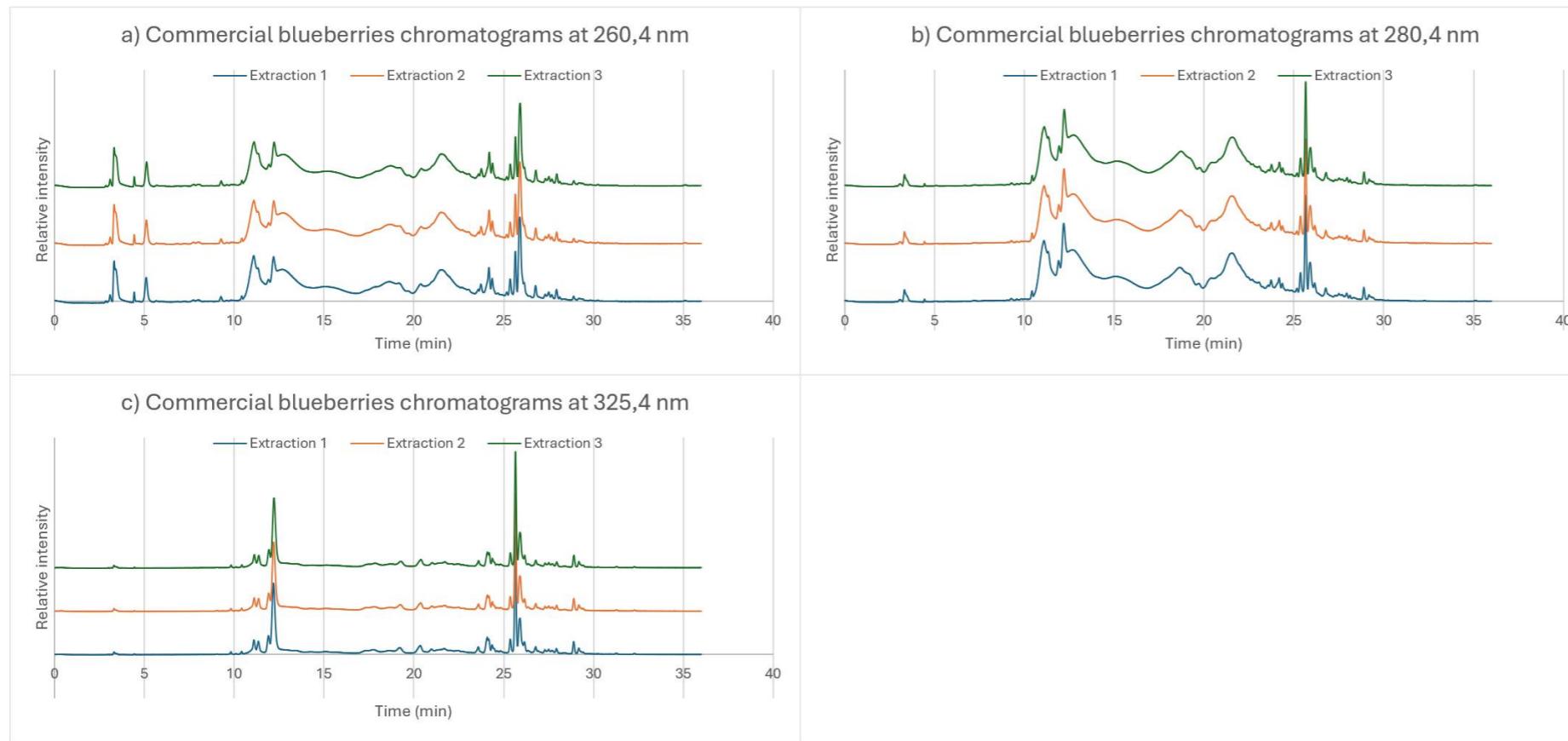


Figure A14. Obtained chromatograms for the 3 extractions done in commercial blueberries sample.

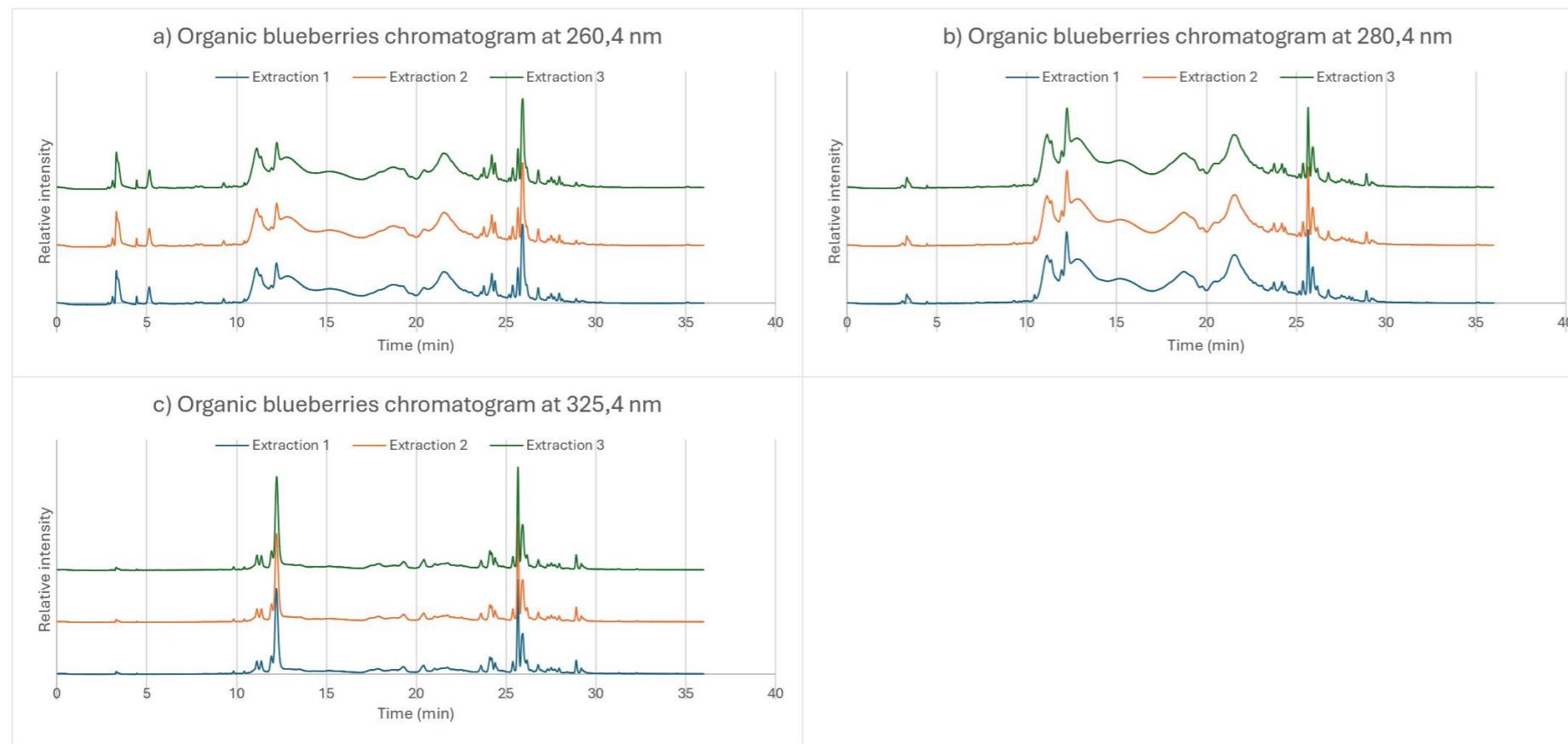


Figure A15. Obtained chromatograms for the 3 extractions done in organic blueberries sample.



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